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**14. ABSTRACT**

Immune-based therapy for solid tumors is a promising area of research, providing the potential for cell-mediated immunotherapies to provide long-lived protection against various stage cancer. Unfortunately, even the most successful clinical trials using T cells or dendritic cells (DC) only show objective response rates in <50% of patients. This is due, in part, to a variety of tumor-derived immunosuppressive mechanisms that arise in cancer patients, rendering antitumor immune responses ineffective. In addition, epidemiological studies have demonstrated that obese individuals face an increased risk of developing cancers, including breast cancer. The reasons for this are likely complex and multi-factorial, but a state of generalized immune suppression may contribute to these findings. Regardless of the body-mass index of the patient, successful long-term treatment of breast cancer must not only reduce the localized tumor burden, but must also target undetected or known metastases that may exist at the time the primary tumor is identified and treated.

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## INTRODUCTION

Breast cancer affects more women than any other single type of cancer. New treatment options for women with metastatic breast cancer are needed, and several types of immunotherapy are currently being investigated as treatment options for advanced or metastatic breast cancer. Unfortunately, even the most successful immunotherapy-based clinical trials only show objective response rates in <50% of patients, which is partially due to a variety of tumor-derived immunosuppressive mechanisms that arise in cancer patients, rendering antitumor immune responses ineffective. In addition, epidemiological studies have demonstrated that obese individuals face an increased risk of developing cancers, including breast cancer. The studies outlined in the funded DOD grant application were designed to test the hypothesis that when compounded by obesity and its associated chronic inflammation, solid tumor outgrowth will lead to the formation of regulatory DC at tumor-distal sites, such as the spleen, which will profoundly affect the generation of TRAIL-induced antitumor immunity. Our interest in the present application to investigate the impact of obesity and chronic inflammation on the therapeutic efficacy of Ad5-TRAIL is quite clinically relevant, and the utilization of a preclinical model that addresses advanced breast cancer (with metastases) targets a potentially increasing number women in the U.S. that could be facing the issues of obesity-induced co-morbidities associated with breast cancer.

## BODY

### Statement of Work (as listed in the original proposal)

The *objective of this application* is to understand how obesity and chronic inflammation affect the therapeutic potential of a recombinant adenovirus encoding the cDNA sequence for murine TNF-related apoptosis-inducing ligand (TRAIL; Ad5-TRAIL) in the treatment of metastatic breast cancer.

The majority of the work is taking place at the University of Minnesota, under the direction of Dr. Griffith. Some of the proposed work in Aim 2 (as indicated) is being conducted by Dr. Lyse Norian at the University of Iowa through subcontract. This division of labor was agreed upon by Drs. Griffith and Norian prior to Dr. Griffith's relocation to the University of Minnesota.

**Task 1. Determine the extent to which obesity and systemic inflammation affect TRAIL-based immunotherapy in breast tumor-bearing mice**

Evaluate the impact of obesity and chronic inflammation on Ad5-TRAIL-based immunotherapy.

1. Analysis of tumor outgrowth after immunotherapy – months 1-12
2. Assess cytokine, chemokine, and adipokine expression as an indication of obesity and chronic inflammation – months 1-12 (serum samples will be taken from the mice used above)

*Outcomes and deliverables from this phase of the project:* Determine the efficacy of Ad5-TRAIL/CpG therapy in mice with advanced breast cancer (primary tumor and metastases). Determine the extent to which obesity and chronic inflammation alter the therapeutic potential of Ad5-TRAIL/CpG therapy in mice with advanced breast cancer.

**Task 2. Identify molecular and functional alterations in DC that arise during tumor outgrowth in obese mice, with particular focus on those properties that would promote tumor outgrowth and metastasis**

A. Assess DC subset phenotype and percentages via multiparameter flow cytometry – months 10-13 (Dr. Norian)
B. Evaluate shifts in DC stimulatory vs. regulatory function – months 12-16 (Dr. Norian)
C. Evaluate pro-tumorigenic DC cytokine production via Multiplex – months 16-20
D. Determine the effects of adipocyte-derived cytokines on DC phenotype and function – months 16-20 (Dr. Norian)

*Outcomes and deliverables from this phase of the project:* The above tasks test the hypothesis that in obese mice with metastatic breast cancer, both TIDC and systemic DC will undergo changes in stimulatory capacities and cytokine production, resulting in their function as regulatory cells that simultaneously inhibit T cell function and promote tumor metastasis.

Relocation of my laboratory from the University of Iowa to the University of Minnesota in August 2011 required the DOD award to be relinquished by the University of Iowa and transferred to the University of Minnesota. This was completed, and the award started at the University of Minnesota April 1, 2012. The following data have been completed to date:

Results pertaining to Task 1.

*Altered serum cytokine profile in female BALB/c diet-induced obese (DIO) mice*

The majority of prior studies on murine DIO used the C57Bl/6 strain, as these mice rapidly become obese after being placed on HFF[1-6]. As our goal was to determine the combined effects of breast tumor outgrowth and DIO on DC function, our experimental model necessitated using the BALB/c strain for challenge with the 4T1 murine breast tumor cell line. We found that BALB/c mice are more resistant to DIO than C57Bl/6 mice, and BALB/c mice placed on HFF for 10 weeks did not develop the systemic inflammation that normally accompanies DIO. Consequently, we modified our protocol so that BALB/c mice were fed HFF for 20 weeks, and performed a thorough characterization of the resulting DIO mice.

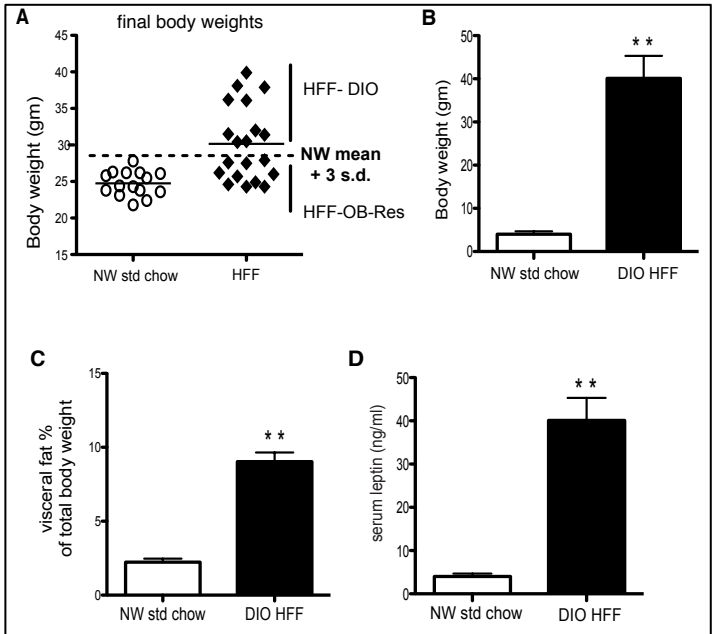


**FIGURE 1.** BALB/c DIO mice show characteristic hallmarks of obesity. *A*, Final body weights for individual mice are shown after being on either standard chow (NW) or HFF for 20 weeks. The dashed line indicates 3 s.d. above the NW mean. *B*, Mean +/- SEM final body weights for 13 NW and 13 DIO HFF mice. *C*, Mean +/- SEM visceral body fat, as a percentage of total body weight, for the same mice used in *B*. *D*, Individual serum leptin concentrations as determined by ELISA, for n= 7 NW, 7 DIO, and 5 OBR mice. ** $p \leq .01$.

We observed that 45-55% of BALB/c mice on HFF showed increased weight gain relative to age-matched mice fed standard chow ("NW" mice) over the same period of time (Fig. 1*A*). Therefore, we defined DIO mice as those having a final weight greater than the mean weight plus 3 s.d. of age-matched NW mice that had been fed standard chow for 20 weeks. The mean body weights of one cohort of 13 NW and 13 DIO mice is shown in Fig. 1*B*. Compared to NW mice, DIO mice had increased percentages of visceral body fat and increased concentrations of serum leptin (Fig. 1*C* and *D*), both of which are hallmarks of obesity.

We next measured the amount of 35 individual cytokines and chemokines in the serum of NW and DIO mice via multiplex array. Of these, only IL-5 and VEGF were elevated in NW vs DIO serum (Table 1).

Table I. Serum cytokine and chemokine profiles for NW versus DIO mice. Serum was harvested from NW or DIO mice (n= 6 NW, n= 7 DIO) after 20 wks on feed, frozen, and analyzed via MultiPlex array for the above analytes.

| Serum Analyte (pg/ml) | NW mean +/- SEM | DIO mean +/- SEM | p < .05 |
|---|---|---|---|
| IL-1α | 51.0 +/- 15.6 | 328 +/- 241.8 | * |
| IL-1β | 1.0 +/- .71 | 3.4 +/- 1.5 | |
| IL-2 | 1.5 +/- .71 | 14.3 +/- 5.6 | * |
| IL-3 | 6.8 +/- 1.1 | 19.3 +/- 13.5 | |
| IL-4 | 6.5 +/- 5.0 | 10.8 +/- 5.7 | |
| IL-5 | 41.5 +/- 2.8 | 23.7 +/- 6.2 | * |
| IL-6 | 35.0 +/- 7.1 | 26.0 +/- 7.3 | |
| IL-7 | 7.5 +/- 2.1 | 55.8 +/- 11.9 | * |
| IL-10 | 4.0 +/- .71 | 15.3 +/- 9.3 | |
| IL-12p40 | 12.3 +/- .35 | 27.6 +/- 11.1 | * |
| IL-12p70 | 4.3 +/- .71 | 8.6 +/- 6.8 | |
| IL-13 | 56.0 +/- 11.0 | 105 +/- 23.7 | * |
| IL-15 | 28.8 +/- 1.4 | 98.5 +/- 26.9 | * |
| IL-17 | 3.0 +/- 1.4 | 37.1 +/- 15.8 | * |
| eotaxin | 13391.8 +/- 612.7 | 18897 +/- 875.4 | * |
| G-CSF | 538.0 +/- 21.2 | 552 +/- 54.0 | |
| IFNγ | 6.0 +/- 4.6 | 31.5 +/- 7.1 | * |
| IP-10 | 711.3 +/- 1.06 | 1726 +/- 510 | * |
| KC | 1208 +/- 205.1 | 1156 +/- 246.5 | |
| LIF | 10.0 +/- 2.1 | 52.9 +/- 13.0 | * |
| LIX | 5731 +/- 1289.1 | 13461 +/- 2038 | * |
| TNFα | 2.5 +/- .71 | 13.5 +/- 9.16 | * |
| VEGF | 3011 +/- 4141 | 184.9 +/- 291 | * |

In contrast, a large number of analytes showed statistically significant increases in DIO vs NW serum, including IL-1α, IL-7, IL-15, IL-17, IFNγ, IP-10, LIF, LIX, and TNFα. Together, these data illustrate that placing BALB/c mice on HFF for 20 weeks generates a robust obesity characterized by systemic inflammation.

*Efficacy of Ad5-TRAIL/CpG ODN therapy against 4T1 tumors*

We examined the ability of Ad5-TRAIL/CpG ODN therapy to restrict the outgrowth of 4T1 tumors implanted into BALB/c mice. Treatment of tumor bearing lean ("NW") mice occurred 7 d after implantation of 4T1-luc. When examined 7 d after therapy, mice treated with Ad5-TRAIL/CpG ODN had significantly lower tumor burden compared to mice treated with PBS. Ad5TRAIL/CpG ODN or PBS injection was directly into the tumor (Fig. 2). As we have had a limited number of mice on the HFF diet meet the above criteria for obesity, we have not yet been able to examine the efficacy of the immunotherapy in 4T1-bearing DIO mice. It is expected that those experiments will begin in the next several months when cohorts of DIO mice become available.

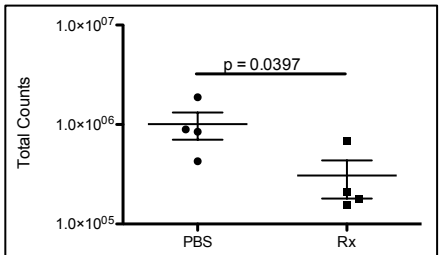


**FIGURE 2.** Ad5-TRAIL/CpG therapy (Rx) reduces tumor burden compared to PBS treatment.

Results pertaining to Task 2.

*Increased percentages of conventional splenic DC in DIO mice*

As a prior report had shown increased percentages of CD11c$^{+}$/ MHC II$^{+}$ splenic DC (spDC) in leptin-deficient *ob/ob* C57Bl/6 mice[7], we examined the frequencies of steady-state spDC in BALB/c DIO mice. Conventional spDC are identified by high CD11c expression, which differentiates them from other cell populations that can express intermediate to low levels of this integrin[8,9]. Fig. 3A shows the gating strategy used to identify spDC. We observed significantly increased percentages of CD11c$^{high}$ spDC in DIO mice (Fig. 3B). An analysis of CD11c$^{high}$ DC subsets revealed equivalent percentages of CD8$\alpha^{+}$ DC, CD4$^{+}$DC, CD11b$^{+}$ DC, and CD8$^{-}$/CD4$^{-}$ DC in the spleens of NW and DIO mice (Fig. 4A). Of note, total live splenocyte counts were nearly identical in NW and DIO mice (Fig. 3C), indicating that the obesity-associated inflammation had not resulted in an overall increase in splenic cellularity. Thus, our findings regarding spDC in BALB/c DIO mice support those reported earlier by Macia et al.[7].

During our analysis of conventional spDC, we noticed that DIO mice appeared to have increased percentages of CD11c$^{low}$/ MHC II$^{+}$ splenocytes, consistent with a plasmacytoid DC phenotype[10]. Further analysis revealed that DIO mice had significant increases in the percentages of plasmacytoid DC as compared to NW mice (Fig. 4B*)*. As our primary focus here was on conventional DC subsets in tumor-free and tumor-bearing mice, we did not investigate the pDC subset further.

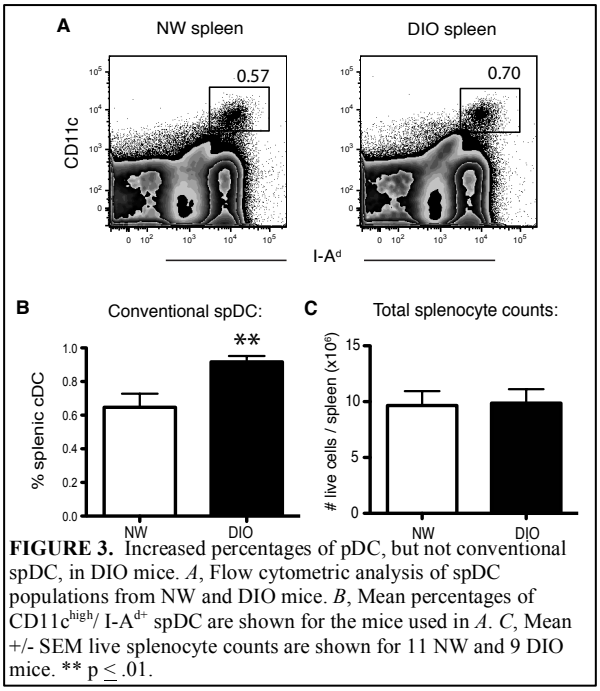


**FIGURE 3.** Increased percentages of pDC, but not conventional spDC, in DIO mice. *A*, Flow cytometric analysis of spDC populations from NW and DIO mice. *B*, Mean percentages of CD11c$^{high}$/ I-A$^{d+}$ spDC are shown for the mice used in *A*. *C*, Mean +/- SEM live splenocyte counts are shown for 11 NW and 9 DIO mice. ** $p \leq .01$.

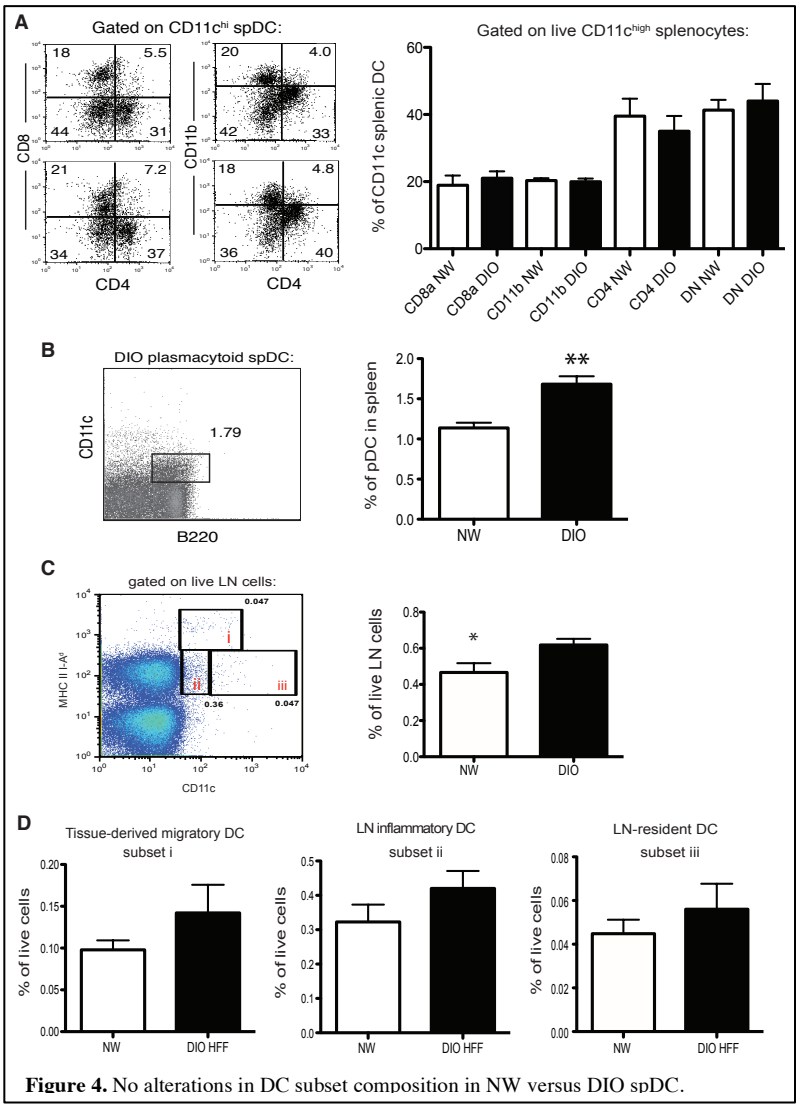


**Figure 4.** No alterations in DC subset composition in NW versus DIO spDC.

A. The mean percentage (+/- SEM) of indicated spDC subsets is shown, where n= 3-4 individual mice per group, pooled from 2 independent experiments. No statistical differences were observed between NW and DIO percentages for any spDC subset examined. B. Statistical increases in splenic pDC from DIO mice, where n = 5 mice per group. C. Increased percentages of total LN DC in DIO mice, without significant alterations in LN DC subset composition. One representative dot plot is shown to illustrate gating strategies. Upper right plot: mean values (+/- SEM) for the percentage of LN DC present, where n= 7 NW and 4 DIO mice. Lower bar graphs indicate the mean percentage (+/- SEM) of LN cells falling within the gates shown in A. * indicates $p < 0.05$.

Lymph node (LN) DC subset composition has not been thoroughly examined in DIO mice. We isolated brachial, axillary, and inguinal LNs from NW and DIO mice, and determined the frequencies of total DC in pooled LNs, as well as percentages of the three main DC subpopulations present, based on expression of CD11c and MHC II (I-$A^d$). Tissue-derived migratory DC are defined as $CD11c^+$/ MHC $II^{high}$ (subset i), inflammatory DC as $CD11c^+$/ MHC $II^+$ (subset ii), and LN-resident DC as $CD11c^{high}$/ MHC $II^+$ (subset iii) (Fig. 4*C*)[11]. DIO mice had increased percentages of total LN DC as compared to NW mice (Fig. 4*C*). No statistically significant alterations were observed in the percentages of any of the LN subsets in DIO vs NW mice, although there was a trend in each case toward increased percentages with obesity (Fig. 4*D*). Together, these results show that DC subset composition is similar between NW and DIO mice, but that greater percentages of conventional spDC, plasmacytoid spDC, and LN DC are present in DIO mice.

*Decreased stimulatory capacity but increased CXCL10 production in DIO spDC*

Previous investigations into the effects of obesity on DC stimulatory capacity produced conflicting results. Studies on naive animals had found that bulk splenic APC from DIO mice and bone-marrow-derived DC from *ob/ob* mice were less able to stimulate naive T cell proliferation than were cellular counterparts from NW mice[7,12]. In contrast, another report found that during influenza infection, lung DC from DIO mice retained the ability to induce IFNγ production in T cells[1]. Thus, our next set of experiments addressed to what extent DIO impacted the steady-state stimulatory capacity of highly purified spDC.

spDC were sort-purified from NW or DIO mice by gating on $CD45^+$/ $Gr\text{-}1^{neg}$/ $CD11c^{high}$/ $CD11b^+$ cells. Following purification, spDC were pulsed with tERK peptide and used to

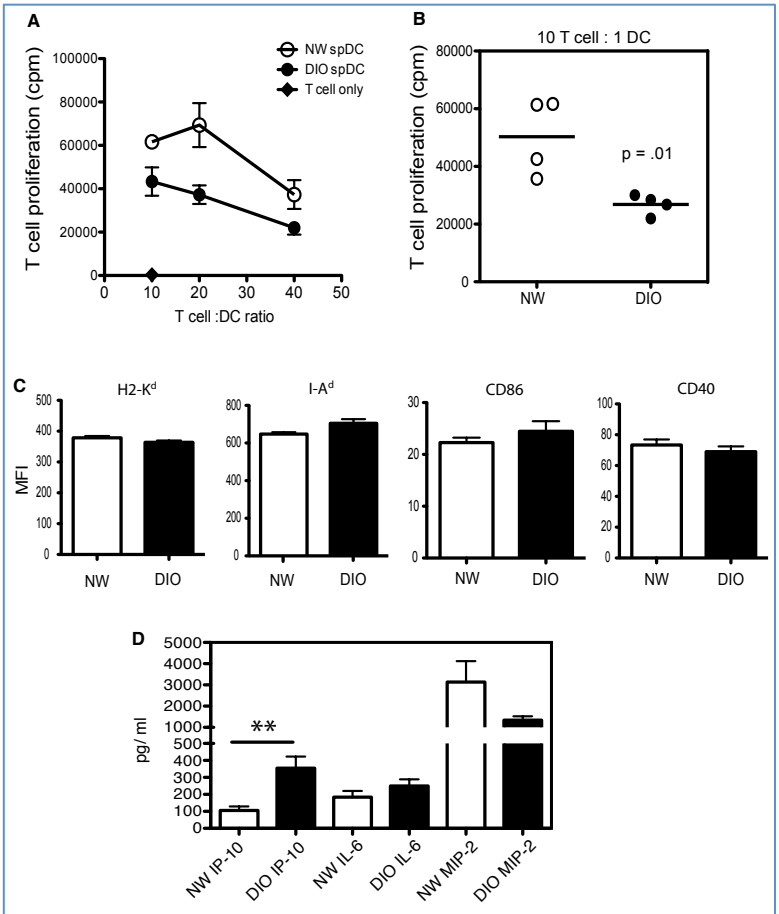


**Figure 5.** Decreased stimulatory capacity but increased IP-10 production in DIO spDC. **A.** Individual results from one independent T cell proliferation assay using sort-purified, tERK-pulsed spDC and enriched $CD8^+$ DUC18 T cells. The mean ± SEM for triplicate wells is shown. **B.** Individual results from four independent T cell proliferation assays, performed as in A, are shown. Bars indicate the mean T cell proliferation calculated. The DIO mean is 58% of the NW mean. **C** Mean fluorescent intensities (MFI) of the indicated surface proteins on gated live $CD11c^{high}$/I-$A^d$ spDC are shown. Bars indicate mean ± SEM. **D** Cytokine and chemokine concentrations are shown for six NW and six DIO spDC samples, obtained from individual mice. Analyte concentrations were determined via Multiplex array. $^{**}p \leq 0.01$.

stimulate $CD8^+$ TCR-transgenic DUC18 T cells *in vitro*[13-16]. The results of 4 independent experiments illustrate that spDC from DIO mice induced less T cell proliferation than their NW counterparts (Fig. 5*A* and *B*). Overall, mean T cell proliferation in the presence of DIO spDC was 58% of that seen with NW spDC.

We next examined DIO and NW spDC to determine if obvious phenotypic differences might account for the decreased stimulator capacity observed in DIO spDC. Bulk splenocytes from NW and DIO mice were stained as indicated (Fig. 5*C*). Interestingly, gating on the $CD11c^{high}/CD11b^+/I\text{-}A^{d+}$ spDC population revealed no significant alterations in surface expression of MHC I, MHC II, CD86, or CD40 between NW and DIO mice. We then asked if DIO spDC possessed an altered cytokine/chemokine profile as compared to NW spDC, since DC-derived cytokines can profoundly alter T cell differentiation outcomes[17]. Following an overnight DC culture with LPS, supernatants were harvested from either DIO or NW spDC cultures, and analyzed via multiplex array for a panel of 24 cytokines and chemokines. The only significant difference found was in the production of the chemokine IP-10, which was higher in DIO spDC than NW spDC (Fig. 5*D* and Table I). For many analytes, such as IL-6, the expression patterns were nearly identical between the two groups, whereas for others, such as MIP-2, trends were visible but did not reach statistical significance. Overall, while the phenotypes and chemokine and cytokine production in NW and DIO spDC were largely comparable, the major difference we detected was a decrease in DIO spDC stimulatory capacity.

*Decreased DC response to CpG stimulation*

Dendritic cells (DC) are key regulators of T cell immunity, therefore normal DC function is essential for achieving T cell-mediated tumor clearance. Given that our immunotherapy includes the TLR9 ligand CpG oligodeoxynucleotide (CpG ODN), we examined DC from lean and DIO mice to see if there was any difference in the response of these cells to CpG ODN simtulation. DC are a heterogeneous population of APC that are essential in the generation of tumor-specific T cell responses[18]. It is now appreciated there are distinct DC subsets with specialized functions. CD8α DC and plasmacytoid DC (pDC) can contribute to the priming, activation, and function of antitumor CD8 T cells. CD8α DC cross-present Ag derived from exogenous sources (e.g., apoptotic cells) in MHC I to CD8 T cells[19], and pDC express TLR9 and produce IFN after CpG ODN stimulation[20]. When we examined the ability of CpG ODN to activate splenic CD8α DC and pDC from lean, DIO, and ObR mice, there was no difference in CD86 expression on unstimulated splenic CD8α DC and pDC after 48 h *in vitro* culture (Fig 6; open symbols).

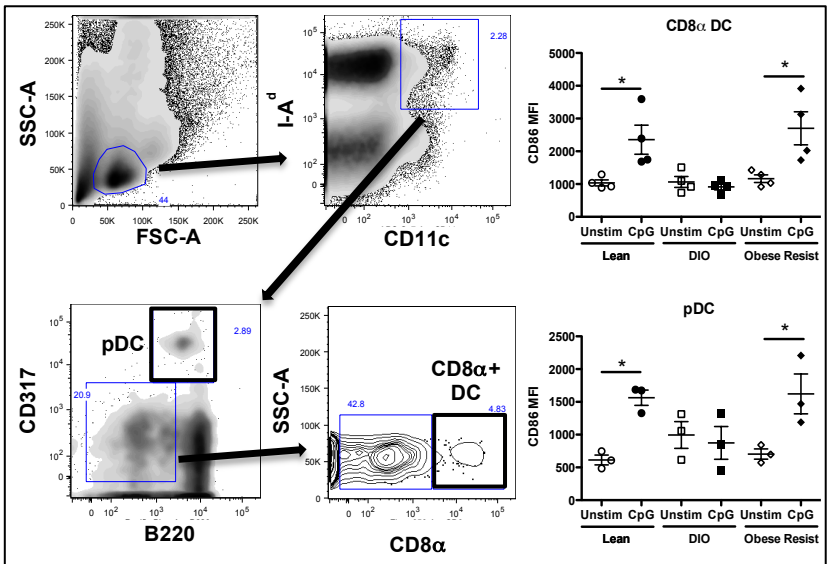


**Figure. 6. CpG does not activate splenic CD8α DC or pDC from DIO mice *in vitro*.** Bulk splenocytes from lean, DIO, and obese resistant mice were cultured *in vitro* for 48 h in media alone or media containing CpG. CD86 expression was determined on CD8α DC and pDC within the bulk cells. The gating strategy used to identify these DC populations is on the left. Averaged CD86 MFI data is on the right. * $p < 0.05$.

However, *in vitro* stimulation of bulk splenocytes from these 3 groups of mice with CpG ODN for 48 h found significantly increased CD86 expression on 'lean' and 'ObR' CD8α DC and pDC *– but not on 'DIO' CD8a DC and pDC* (Fig 6; closed symbols).

*DC infiltration into 4T1 breast tumors*

We examined whether there was any difference in DC infiltration into 4T1 tumors implanted into NW and DIO mice. For these experiments, mice were challenged with 4T1 tumors, and the tumors and spleens were harvested at the indicated times. The frequency of $CD11c^{hi}$ $CD11b^{+}$ $I\text{-}A^{d+}$ DC within the tumors were determined by flow cytometry. Figure 7 shows no significant difference in the frequency of DC within the tumors from NW and DIO mice. There was a transient increase in the frequency of DC within the spleens of DIO mice 14 d after tumor challenge, but this difference was no longer evident on d 21.

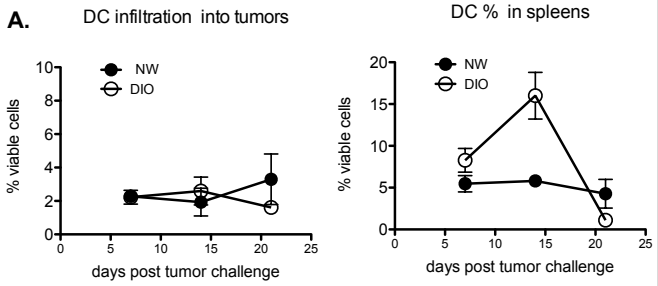


**Figure 7.** No difference in DC infiltration into tumors in NW or DIO mice. NW and DIO mice were challenged with 4T1 tumors on day 0. Tumors and spleens were harvested at indicated times and stained for CD11c, CD11b, I-Ad. The percentages of DC are indicated. n=7 mice / group

*Effect of adipocytes on DC phenotype*

Since adipocytes produce an array of molecules with immunomodulatory potential, we examined the extent to which adipocytes from NW or DIO mice altered DC phenotype. Thus, DC were isolated from MW or DIO mice and placed into culture with the indicated populations of adipocytes. After 48 h, the phenotype of the DC was analyzed by flow cytometry with respect to $I\text{-}A^{d}$ and CD83 expression (Figure 8). No differences in DC phenotype were seen.

B.

| | | Adipocytes added: | | |
|---|---|---|---|---|
| | | None | DIO | NW |
| % $I\text{-}A^{d+}$ DC: | DIO DC | 80.8 | 86.5 | 85.3 |
| | NW DC | 93.4 | 88.3 | 81.8 |
| % $CD83^{+}$ DC: | DIO DC | 1.6 | 2.6 | 1.1 |
| | NW DC | 1.4 | 2.4 | 1.9 |

**Figure 8.** DC were isolated from DIO or NW mice and placed into culture with the indicated populations of adipocytes. After 48 hr, DC were stained for I-Ad and CD83. No differences in DC phenotype were present.

## Key Research Accomplishments

- 4T1 tumor progression is suppressed in lean mice after Ad5-TRAIL/CpG therapy
- Establishment of diet-induced obesity model
- DC function is suppressed in DIO mice

## Conclusions

Our data to date suggests DC function is impaired in DIO mice compared to lean (NW) mice. This observation has significant implication with regard to the potential success of tumor immunotherapy that relies on DC function to phagocytize apoptotic tumor cells, process/present

tumor antigen to T cells, and/or respond to immunotherapy components (i.e. CpG) to mature and produce cytokines needed for a maximal T cell response. Based on these data, we expect the Ad5-TRAIL/CpG immunotherapy will be unable to suppress 4T1 breast tumor outgrowth in DIO mice. This observation has significant impact on the future development of immunotherapy protocols for breast cancer that would be used in obese patients. Understanding the defects in the DIO setting will be essential in identifying additional agents to the immunotherapy protocol to overcome the immune system defects present in the face of obesity.

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